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## Regulation of protein homeostasis in acute and chronic stress

Wu, Di

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## **Chapter 1**

### **Introduction and aim**

**Abstract:**

The balance between protein production, folding, transport, assembly and the timely degradation of proteins is defined as protein homeostasis. This process is controlled by a network of protein quality control (PQC) that includes molecular chaperone and protein degradation systems. Here, we will review the pathways and components that regulate protein homeostasis in eukaryotes, with a focus on the network of heat shock proteins (Hsps).

Various situations may perturb protein homeostasis. We will review how acute injury and chronic diseases may lead to protein homeostasis imbalances. Furthermore, it will be eluted how, under each of these conditions of stress, heat shock proteins may help to rebalance protein homeostasis, preserve cell and tissue integrity, and maintain organismal vitality.

The experimental research in this thesis is dedicated to two different challenges: 1) poultry breeding industry where animals often suffer from acute heat stress induced myocardial cell injury; 2) heritable neurodegenerative diseases in humans, in particular Huntington's disease (HD), where patients chronically express an aggregation-prone mutant protein. Our results suggest that these different forms of stress may require different set of chaperones and different pathways to induce them for rebalancing protein homeostasis.

## **Protein homeostasis**

The balance between protein production, folding, transport, and assembly and the timely degradation of proteins is defined as protein homeostasis (1, 2).

### ***Physiological regulations of Protein homeostasis***

For proteins to become functionally active, they must be folded into specific 3D structures during or after emerging from ribosomes as linear polypeptide chains (3, 4). Whereas folding is primarily driven by the amino acid sequence (the Anfinsen principle (5)), folding towards the native conformation within the dense environment of the cell can be non-productive and lead to non-native protein-protein interactions (aggregates) that not only lead to non-functional proteins (loss-of-function) but that also may exert toxic effects (gain-of-toxicity) in cells (6-10). So, in vivo, protein folding requires assistance by molecular chaperones. The largest class of such molecular chaperones are the proteins from the various heat shock protein families. These will be further discussed in part 2 of this review.

When folded, protein conformations are constantly challenged by endogenous or exogenous stress conditions which can lead to (partial) protein unfolding, again increasing the risk of aggregation, endangering cellular viability, tissue integrity and ultimately organismal fitness. Those unfolded proteins increase the need for chaperones (see 3.1) to prevent their aggregation or to disaggregate them for subsequent refolding or degradation (2). Indeed, acute proteome challenging stresses can turn on stress response pathways (unfolded protein responses) that ensure the required re-balancing of protein homeostasis (see 3.3.1).

### ***Folding***

Proteins are synthesized as linear amino acid chains which need to fold into secondary, tertiary and quaternary structures to become functional (3, 4). Protein folding is driven primarily by the side chains of the amino acids (the Anfinsen principle (5)) but in cells require molecular chaperones for assistance (11). In regulated cycles of binding and release, molecular chaperones assist immature proteins along their folding landscape to the native state without being part of the final folded and functional complex (11). At the same time, such cycles of interaction also prevent off-pathway reactions, e.g. formation of toxic aggregates that hall mark several degenerative diseases (5).

### ***Translocation***

Besides being involved in folding, chaperones also assist in the transport of proteins between different cellular compartments (11). Protein translocation can happen co-translationally or post-

translationally. Some chaperones are related to the post-translational protein translocation. For example, Hsp70 can mediate the translocation of proteins to mitochondria matrix (12). It requires the substrates to be in a largely unfolded state. Hsp70 binds such and them across the mitochondria via the Tom70 receptor which is on the outer mitochondria membrane (13, 14).

#### *Protein complex assembly and disassembly*

In many cases, specific biology functions are exerted by protein complexes composed of multiple individual proteins, for example, proteasome complex. 26S proteasome is composed by a 20S and a 19S subunit. The assembly of each subunit needs proteasome assembly chaperones (PAC1-4) in human (15). These chaperones can prevent aberrant dimerization of  $\alpha$ -ring and ensure  $\alpha$ -subunit and  $\beta$ -subunit are correctly incorporated (16, 17).

Chaperones also play a role in protein complex disassembly. For example, the DNAJ family member auxilin (DNAJC6) and Hsc70 (HspA8) which is the constitutively expressed Hsp70 (18, 19) mediate the disassembly of clathrin-coated vesicles in the uncoating of clathrin which is important to form coated vesicles (18).

#### *Degradation*

When proteins cannot be re-folded (e.g. due to mutations) or are no longer needed, they must be disposed. The most important protein degradation pathway in eukaryotes are ubiquitin-proteasome system (UPS) and autophagy (20).

#### *UPS*

For the discovery of UPS, Aaron Cheichanover was awarded Nobel Prize in chemistry in 2004 (21). Substrate proteins in eukaryote that need to be disposed are labeled with ubiquitin first. Ubiquitylation of substrate proteins involves three enzymes: an E1 ligase that will activate the monomer ubiquitin (22), an E2 enzyme that will transfer the ubiquitin from the E1 and conjugate it to its own active site. The E2 can next transfer to E3 ligase with specific bounds substrates and next this E3 ligase will ligate the ubiquitin to the substrate (23-25). Ubiquitylated substrate can next be delivered to proteasome, a multi-protein complex consisting of 19S and 20S (24, 26). The 19S subunit is an AAA ATPase that can transfer the substrate to and open the entrance of the 20S subunit which contains the protease activities to digest the substrate (26, 27). Ubiquitin is a conserved polypeptide composed by 76 amino acids (28).

Chaperones are thought to connect to the UPS in different manners. First, by preventing aggregation during stress, they maintain proteins in a UPS-degradation competent state (29). In addition, certain co-chaperones (e.g. DNAJB2) directly connect to the proteasome and when bound to its clients, it favors

the UPS-mediated degradation rather than their refolding (30). In doing so, DNAJB2 cooperates with Hsp70, its co-chaperones BAG-1 and the carboxyl terminus of Hsc70-interacting protein (CHIP) which has E3 ubiquitin ligase activity (31). In another word, binding of specific DNAs to un- or misfolded clients, together with other co-factors, can be important for client fate (32). Inversely, the state of the client may dictate which specific co-chaperones binds and this may determine its fate. An elegant example of the latter model is that of the ER resident HSP70 machine. Herein, ERdj3 and Bip, that promote client folding, recognize a diversity of sequences throughout substrates that differ from more rare sites recognized by Grp70, ERdj4 and ERdj5. These binding sites are predicted to have tendency to aggregate (33). Once being recognized and bound by Grp70/ERdj3/ERdj5, these potential aggregation-prone substrates are directed to ER associated degradation (ERAD) (33). This is consistent with earlier findings connecting ERdj4 and ERdj5 to proteasomal degradation (34-36).

### *Autophagy*

The other main degradation pathway that cells used to dispose proteins is the autophagosomal-lysosomal pathway, for which discovery Yoshinori Ohsumi was awarded the 2016 Nobel Prize in Physiology or Medicine (37). There are three forms of autophagy: microautophagy, chaperone-mediated autophagy, and macroautophagy (38, 39). Microautophagy entails the direct engulfment of cytoplasmic material into the lysosome (40). Chaperone-mediated autophagy involves the recognition of cargo proteins by HspA8 and subsequent delivery of the cargo into the lysosome (41). In macroautophagy, a bilayer lipid membrane is formed around the cargo (damaged proteins, protein aggregates and damaged organelles) to generate so-called autophagosome that will fuse with the lysosome to form autophagy-lysosome in which multiple hydrolase digest substrates into small molecules for re-use or be released from cell (42, 43).

Like for UPS, some chaperone actions are connected to macroautophagy. This includes e.g. the complex of the HSPB8, HSP70 and its co-chaperone BAG3 (44). Under conditions of proteasomal overload, cells up-regulate HspB8 and BAG3. As a result, BAG3 competes with BAG1 for Hsp70 with its bound substrates and these substrates are now no longer delivered to the proteasome, but rather end up in autophagosomes (45). Also the action of HspB7, another member of the sHsp family (see below) has been connected to autophagosomal degradation (46, 47).

## **Hsps in protein homeostasis**

As mentioned above, molecular chaperones play a crucial role in maintaining protein homeostasis, not only by guiding folding, transport, and assembly but also by assisting in the degradation of proteins. Heat shock proteins form the largest class of molecular chaperones in the cell.

These Hsps can be divided into different families: HspA (Hsp70), HspB (small Hsp), HspC (Hsp90), HspD (Hsp60/Hsp10), CCT/TriC and HspH (Hsp110) and DNAJ (Hsp40) families (48). These different Hsps families have no structural similarities, but within families multiple members are present that share features characteristic of their family but can contain with variable other domains that serve for functional specifications. Globally, 4 categories of chaperones can be distinguished:

- I. The class of sHsps, ATP-independent “holdases” (49).
- II. The ATP-dependent Hsp70 machines, consisting minimally of one Hsp70, one DNAJ and one nucleotide exchange factor (NEF), central to the folding and degradation of most proteins (48).
- III. The ATP-dependent Hsp90 machines, regulated by a multitude of co-factors (50, 51), and required for the docking and maturation of a growing list of important growth factors, transcription factors and hormone receptors (52).
- IV. The class of chaperones, including the mitochondrial Hsp60/Hsp10 and cytosolic TriC complex, required for the folding of a subset of complex and often essential proteins (53).

These categories form an interacting network of protein quality control that guide most proteins from the cradle to the grave. For this thesis, the focus is on the small heat shock protein (sHsp or HspB) that often interact with and provide substrate to the Hsp70 machines. These will be shortly introduced below.

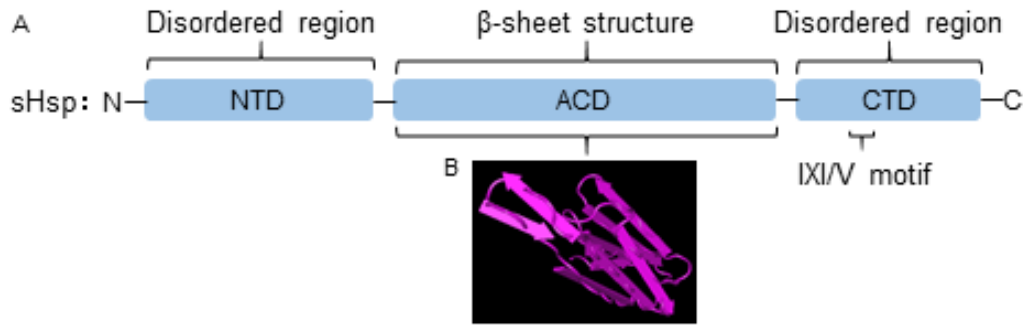
### ***Small heat shock proteins (HspBs or sHsps)***

Small heat shock proteins are ATP-independent chaperones of which there are 10 members in human, HspB1-10 (54). All of them have a conserved  $\alpha$ -crystalline domain (ACD) flanked by variable N- and C-termini (55) (Figure 1).

sHsps exist in almost all living organisms with overlapping structural and functional characteristics, although they can be highly variable. Below, we will mainly refer to mammalian sHsps.

#### *Oligomerization of small heat shock proteins*

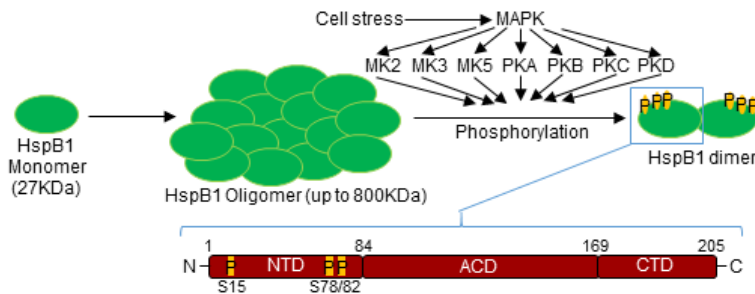
Most of the sHsps can form highly dynamic oligomers (56, 57). The ACD is responsible for assembly of the sHsp dimer while the NTD and CTD are crucial to the stability of the oligomer (55, 58). The ACD contains 94 amino acids on average, forming  $\beta$ -sheet structures. Two homo- or hetero- sHsps form a dimer by the connections between  $\beta$ -sheets. By such bindings, sHsps dimers (or also monomers) can serve as building blocks to form larger oligomers (59). Often, the sHsps not only form homo-oligomers but also hetero-oligomers with one or multiple other members of the family (60). Exceptions are HspB2 and HspB3 that form a quaternary complex (61), HspB7 that does not oligomerize and seems to exist as a dimer only (62) and HspB8 that forms a complex with Hsp70 and BAG3 (63).



**Figure 1, Schematic domain structure of sHsps.**

**A**, Generally, sHsps contains three domains: an N-terminal domain (NTD), an  $\alpha$ -crystalline domain (ACD) and C-terminal domain (CTD). The ACD is the conserved domain characteristic of sHsps and forms a  $\beta$ -sheet structure. Both the NTD and the CTD are variable amongst the different sHsps and generally are largely disordered. There is an IXI/V motif in the CTD of most HspB members, except in HspB6 and HspB8. **B**, 3D structure of human ACD (PDB ID: 2Y22).

The oligomerization of the more canonical sHsps is a very dynamic process and the subunit exchange can be accelerated by stresses such as heat or environmental stresses. Such stress conditions will, amongst others, activate diverse signaling pathways leading to phosphorylation of sHsps, in particular the most studied HspB1 and HspPB5 members (57). Phosphorylated HspB1 will de-oligomerize into dimers (Figure 2). It is thought that this dynamics is crucial to the two main functions of HspB1 and HspB5, being a “holdase” for un- or misfolded proteins and for stabilization of the cytoskeleton (49).



**Figure 2. Schematic graph of phosphorylation mediated HspB1 oligomerization /de-oligomerization.**

Under native condition, HspB1 monomer will form large oligomer. Under stress conditions, HspB1 will be phosphorylated by kinases in downstream of MAPK pathway at Ser15, Ser78 and Ser82. Phosphorylated HspB1 will disassemble and exists as dimer, the activated formation.

### *Holdase functions of small Hsps*

Under acute stress condition, a large amount of protein will unfold and run the risk to form irreversible, noxious aggregates (64). The increase in oligomeric dynamics of sHsps like HspB1 and HspB5 under such conditions is thought to be the first line of defense against such acute forms of stresses, in addition to translation pausing and upregulation of the expression of subset of members within all Hsp families. As such, sHsp oligomers are considered as activate-able chaperone reservoir from which released dimers will bind to the hydrophobic residues of (stress unfolded) client proteins (65). Then, they



will reassemble into larger HspB1-client complexes in which the substrate are maintained in either a folding or a degradation competent. This is the so-called “holdase” function of sHsps (49).

As sHsps do not have ATPase activity, they can “hold”, but cannot spontaneously release their substrates (49) and they require the ATP-regulated Hsp70 machines for client release and further processing (66), which can either be refolding (67) or degradation (68, 69).

Although the above described holdase concept has been established for some decades, the detailed mechanisms of how sHsp bind to and “hold” the their client proteins, how the ATP dependent chaperon recognize, interact and process the “holdase complex”, or how the fate of the unfolded protein in the “holdase complex” is decided still remain to be elucidated. Moreover, if and how less studied members of the family, in particular e.g. the dimeric HspB7, share such a holdase activity has still largely remained elusive.

#### *Cytoskeletal protection mediated by sHsp*

The cytoskeletal network is composed of three distinct elements, being the actin microfilaments (MFs), microtubules (MTs) and intermediate filaments (IFs) (70). These three major cellular filaments not only control cell shape and integrity, but also are crucial to many cellular functions, including intracellular transport and cell division (71, 72).

The cytoskeletal is also easily disrupted under conditions of acute stresses like heat shock. In fact, the protection of such a cytoskeletal collapse is one of the first and best studied function of sHsps (73, 74).

HspB1, HspB5 and HspB6 are HspBs known to be cytoskeletal protective so far (75, 76). HspB5 ( $\alpha$ B-crystallin) can be phosphorylated on serine<sup>59</sup> by p38 which is downstream of RhoK, PKC and PKA, and therefore be activated. The activated HspB5 will associate to intermediate filaments, stabilizing and protecting the integrity of intermediate filaments, preventing aggregation (77, 78). Phosphorylated HspB1 (Hsp27) can also increase stress resistance of actin filament and therefore regulate the dynamic of actin filament (79). A special case is HspB6 which was considered not to interact with actin filaments directly (80) but which is thought to act as an actin-crosslinking protein (76).

#### *Cellular functions related to activities of sHsps*

Related to the two functions of sHsps described above, the (over)expression of some sHsps have been shown to be related to cell motility (81), which requires rapid and dynamic cytoskeletal remodeling, with relevance to e.g. differentiation (82) and cancer invasiveness and metastasis (83). In the eye lens, highly expressed HspB4 ( $\alpha$ -A crystalline) and HspB5 ( $\alpha$ -B crystalline) protect the gamma crystallins from aggregating due to UV-A (84) to prevent cataract. sHsps have also been suggested to buffer against

age related aggregation (85) and indeed in *Drosophila* and *C. elegans* models, Hsp22 can increase organismal lifespan (86-88). Inversely, malfunctions or mutations in sHsps can give rise to cardiovascular diseases (89), and neuronal or muscular disorders (90, 91) that are often associated with protein aggregation. Also, in situations of acute and chronic stresses, sHsps have been implicated to have protective effects. Below, I will first explain what we imply with these terms and shortly summarize what role sHsps have been suggested to Hsps under each of these conditions.

## **Disturbances in protein homeostasis**

Cells can become stressed after exposing to a variety of intrinsic and extrinsic forms of proteotoxicity that can either be acute or chronic (92). Here we review the disturbances in protein homeostasis caused by acute or chronic stress.

### ***Acute stress***

The acute stress here is referred as stress caused by relatively strong environmental changes within a relatively short period such as dramatic temperatures changes, oxidants, UV, chemical reagents or heavy metals. Acute stress generally activate unfolded protein response (UPR) or the heat shock response (HSR) in cell (93, 94). These responses will up-regulate specific chaperones and down-regulate ongoing processes that endangers protein homeostasis (e.g. rapid translation of risky proteome under stress factors) to restore the protein homeostatic balance (95).

### ***The Heat shock response (HSR)***

The HSR is a highly conserved, auto-regulatory transcriptional program which is upregulated by increased proteomic burden in the cytosol and nucleus (96, 97). It was discovered as response to elevated temperatures (98, 99), which is what gave it its name. Nowadays, we know that the HSR can be induced by a multitude of stress factors that either damage proteins or cause large changes in the proteome. In fact, it is one of the most conserved and strongest transcriptional response known (95, 100).

The main transcriptional regulator steering the HSR is a transcription factor, HSF1 (heat shock factor 1). Under normal conditions, the activity of HSF1 is negatively regulated by Hsps (101). Under acute stress conditions, these Hsp's will bind to unfolded proteins, allowing HSF1 to bind to heat shock elements (HSE) which are present in the promoter of heat shock proteins (Hsp's) (102). This leads to the elevated expression of these Hsps, which can bind to and stabilize the cytoskeleton, refold misfolded protein or direct them towards degradation, hereby, restoring protein homeostasis and help cells to survive from the stress (103, 104).

Each family of Hsps has members that are up-regulated upon HSF-1 activation. However, it is important to realize that several members are not (54, 105). The same is true for the sHsp's, of which in humans only HspB1, HspB5, and HspB8 are known to be up-regulated upon several stresses (heat stress, oxidative stress, dramatic changes of pH and exposure to toxic chemical) (106). Evidence that such indeed is relevant to resistance to acute stresses is provided that the single ectopic over expression of each of these members: HspB1 protects against cytoskeletal collapse (79) and enhances protein disaggregation of heat denatured proteins and can therefore protect against heat-induced cell death (57, 107, 108) HspB5 was also known for its cytoskeleton protective function (77, 78). HspB8 can be recruited into stress granules (SGs) which are membrane-less ribonucleoprotein complex induced by acute stress. By forming HspB8-Bag3-Hsp70 complex, HspB8 maintain the function of SGs and direct clients to autophagy (109).

#### *Unfolded protein response (UPR)*

Another cellular response to acute proteomic stresses is the unfolded protein responses (UPR) in reaction to disturbances in the protein homeostasis in the endoplasmic reticulum (UPR<sup>ER</sup>) or mitochondria (UPR<sup>Mit</sup>) (94, 110). Also these responses can be triggered by external stimuli (e.g. hyperthermia, oxidants or other stress factors) or physiological requirements (e.g. B-cell differentiation inducing UPR<sup>ER</sup>) (94).

The UPR<sup>ER</sup> consist of 3 different branches: these are the PERK, IRE-1 and ATF-6 regulated pathways that are each driving a series of cascades to pause translation, to activate specific ER and Golgi chaperones and to enhance degradative processes (activation of ER-associated degradation (ERAD) and autophagy). Relevant to this small HSPs is that so far the only sHsps that is activated by the UPR<sup>ER</sup> is HspB8 (together with its partner BAG3) (109, 111).

The UPR<sup>Mit</sup> is a stress response reacts to the protein homeostasis imbalance in mitochondria. The UPR<sup>Mit</sup> transmits the stress signal to nucleus to promote the expression of nuclear-encoded mitochondrial chaperones such as Hsp60, Hsp10 and mtDnaJ, and the protease ClpP, to rebalance mitochondria homeostasis (112-114). So far, no small HSPs are found in the mitochondria of mammalian cells. But in *Drosophila*, there indeed has at least one mitochondrial sHsp (DmHsp22) which has been shown to have protective effects in oxidative phosphorylation and be able to increase life span upon overexpression (115). Inversely, however, mammalian small HSP have been suggested to protect against mitochondrial-dependent apoptosis (116) and to interrupt the caspase cascade downstream mitochondrial stress (117), suggesting that small HSPs may antagonize the (need of) the UPR<sup>Mit</sup> in this way.

*Translational response to acute stress*

One of the most prominent form of cytoplasmic proteotoxicity caused by acute stress is ribosomal stalling (118). Ribosomal stalling can due to low tRNA abundance, repetitive sequence, impaired co-translational folding, or molecular misreading (stop codon read through) (118). Nascent chains on stalled ribosome are highly prone to form cytoplasmic aggregations (118). Under such condition, a ribosome-associated quality control (RQC) system can be activated (119, 120). Under such situation, the stalled ribosome will be disassembled into 40s and 60s subunit, the later one contains the nascent protein. The nascent protein will then be labelled with poly-ubiquitin by Rqc2 and Ltn1 which is a specific E3 ubiquitin ligase for ribosomal quality control system. The poly-ubiquitylated nascent protein will next be released from 60s subunit and be removed by the degradation pathways (121-123).

In forms of external stress that affect polysomes, another translational response can be activated, which involved the sequestration of mRNA and RNA binding proteins in stress granules (SG) (124). SG are membrane less structures (phase separations) that are thought to store mRNA for a rapid regain of translation to maintain cell-survival after stress (125). Relevant to this thesis is that many sHsps can be recruited into SG as well as other membrane-less granules where they mainly play a role in ensuring the reversibility of the various proteins in such structure, for example, to prevent theses liquid structures to adopt a solid state (124, 126).

*Acute stress in breeding industry*

One of the many potential applications of the HSR is its protective implication in animals breeding industry. One of the most common forms of stress is hyperthermia exposure (127, 128). It was estimated, even in advanced countries like the USA, the annual loss in breeding industry caused by hyperthermia exposure can be as much as 2.4 billion dollars if no appropriate protection is used (129). One of the most impacted branches is chicken breeding. Poultry is more sensitive to high temperature because they are covered by feather, they have no sweat gland, and they are usually raised in crowded environments (129, 130). For example, the heat wave the North America in 2006 resulted in the death of over 700,000 chickens in California alone (131). Acute heat stress injury causes multiple organs failure; especially heart injury is regarded as a main lethal factor caused by hyperthermia exposure (132). Even more, those birds that survived from hyperthermia, had accumulated significant injury and lowered production capacities (133). So, means to alleviate the risk of heat stress injury in animals would have important impact, both for animal welfare and economic reasons.

In this thesis, we attempt to activate the HSR by pharmacological means in order to preventing the following stress caused by hyperthermia in poultry. Below, a short summary on pharmacological activation of the HSR is provided.

### *Pharmacological HSR activators.*

Multiple pharmacological compounds have been identified to induce the HSR (134-138). These chemical compounds can be generally divided as two kinds. The first kind activate HSR by casting mild stress to cells. For example, proteasome inhibitor can increase the level of mis-folded protein in cell, hence, activate HSF1-HSE binding to induce Hsps (139). When condition is optimal, the HSR induced by appropriate amount of proteasome inhibitors, e.g. lactacystin and CEP1612, was reported that protectively suppressed the nuclear inclusion (140). Moreover, a metallic compound, stannous chloride, was found to be able to induce Hsp70 as a protectively anti-inflammatory reagent (141). However, inducing HSR by these stress inducers are apparently risky for reasons of the dosage and drug residue. It's difficult to control every animal in the herd intake the same amount of drug from water or food, therefore, some animals can be poisoned by over-dosage while others might take in insufficiently. It's also difficult to rule out the residual. The accumulation of these chemicals (for example, arsenite, a HspB5 inducer (142-144)) can contaminate animal products and jeopardize human food security. Another idea to activate the HSR is by assisting the expression of Hsps known as co-inducer. One of the typical compound is geranylgeranylacetone (GGA). GGA was developed as an antiulcer, later it was found to be a Hsps inducer that inhibited cell death and polyQ accumulation in spinal and bulbar muscular atrophy (SBMA) (145). Chemical studies reveal that GGA can be bonded by Hsp70, therefore, replace and release HSF1 to bind to HSE and activate Hsps transcription (146). Co-inducers are apparently better solution to protectively induce HSR, but the choices are limited by price, safety and efficiency in breeding industry

However, acetylsalicylic acid (aspirin, ASA), another inducer of HSR has already proven safety as an antipyretic analgesic anti-inflammatory medicine (147), although its mode of activating the HSR has not been elucidated exactly (148). Given its effective Hsps induction function with relatively low side effects and low price (148, 149), we decided to specifically test whether pre-treatment with aspirin can protect chicken myocardial cells from the heat stress (Chapter 2).

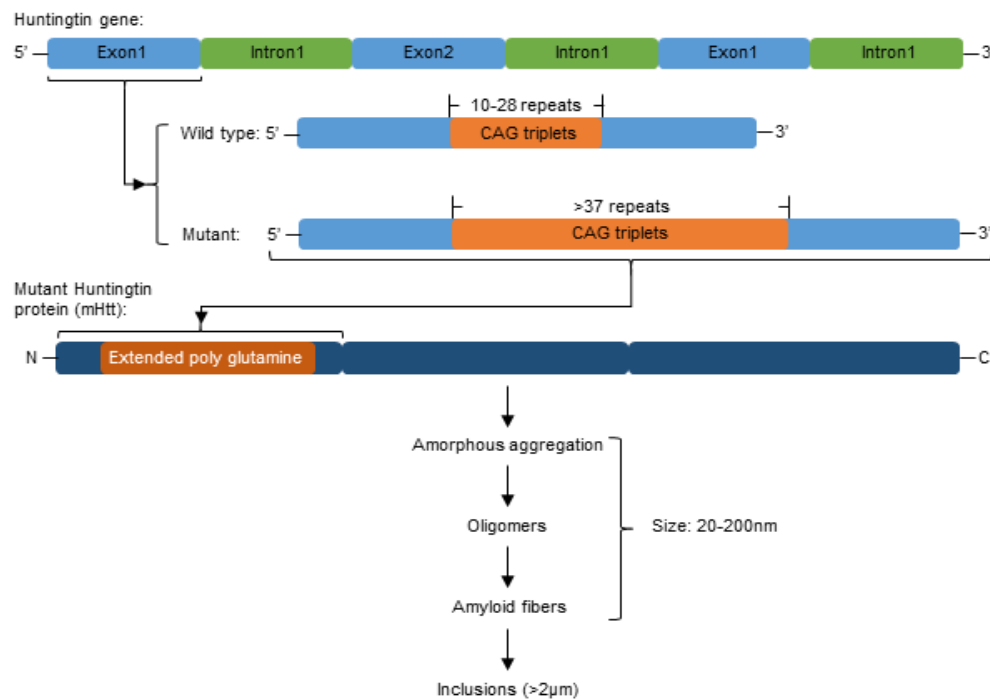
### ***Chronic stress***

With the term chronic stress, we mean to imply long term exposure to relatively mild changes (e.g. expression of mutant polyQ proteins and aging).

#### *The cause of chronic stress*

Genetic mutation can cause loss of function or gain of toxicity in some specific proteins (150, 151). These disease proteins are not toxic enough to cause significant symptoms immediately, but will accumulate with aging until disease onset threshold (92). Several neurodegenerative diseases, for

example, Huntington's disease (HD), can be considered as chronic stress. HD is an autosomal dominant, hereditary neurodegenerative disease, which is caused by the expression of a CAG expansion on the Huntingtin (Htt) gene, leading to an expanded polyglutamine (polyQ) stretch in Htt protein (152) (Figure 2). Wild type Huntingtin (Htt), which exists in almost all cell types in humans, plays an important role in cell signaling and axonal transport (153-155). Targeted disruption of Htt in mice embryo was found to be lethal (153). Rather than a loss-of-function, the disease is thought to be due to a toxic gain-of-function: due to the chronic expression of the mutant Htt polyQ protein tend to form aggregates (152). The longer polyQ stretch is, the more is prone to form such aggregates. Yet, HD patients(156), but also several HD disease models (157) develop normally, are healthy at birth and only develop symptoms upon aging with an earlier disease onset when the expansions are longer (152). This indicates that the chronic exposure to the mutant protein can be dealt with early in life but that this ability to handle the mutant protein gets lost upon ageing.



**Figure 3. Model of Huntington's disease.**

Extended CAG repeats in the Exon 1 of Huntingtin gene encoding extended poly glutamine (polyQ) in mutant Huntingtin protein (mHtt). mHtt can form inclusions larger than 2µm in neuro cells.

As stated, the mHtt tends to aggregate; these aggregates can finally develop into amyloid fibrils and accumulate in inclusion bodies (IBs) (158, 159). There is still a debate on the toxic nature of aggregates, amyloids and IBs. Whereas sequestration of aggregates or amyloids into entities like IBs may have serve as temporal and protective storage (160), this does provide information on what type of aggregates (amorphic or amyloid) are more or less toxic. Clearly, it seems rather that reversibility is a more important determinant, which is now seen for both non-amyloidogenic aggregates (e.g. after acute stress (161)) and

for amyloids (as in chronic stress (162)). Moreover, the position of aggregates, amyloids or inclusion within cells may be more important for toxicity than their precise biochemical nature: e.g. whilst inclusion body in the soma or nucleus of neurons (163, 164), aggregates or inclusions in axonal protrusion (e.g. ballooned neurons:(165)) will have dramatic detrimental consequences for neuronal functioning: indeed axonal trafficking seems one of the early events associated with aggregation in neurodegenerative diseases (166). Finally, the surface characteristics of the diverse forms of aggregates and how they interact with cellular constituents or how they may lead to trapping of essential proteins or protein quality control components may (co)determine their toxic nature (158).

Aggregation can be both the cause and the consequence of the impairment of protein homeostasis (160). On the one hand, the misfolded protein or aggregation will overwhelm the protein quality control (PQC) system which is highly connected to HSF1 (167, 168), and imbalance the protein homeostasis (160). On the other hand, aging can weak the PQC. Then polyQ proteins cannot be degraded in time, and start to accumulate and aggregate (169).

#### *Chronic stress rescue*

Unlike acute stress that rapidly induce multiple stress response pathways, chronic forms of stress usually do not activate these pathways, at least not until most damage has been done (170). Below, we will shortly summarize pathways that control the constitutive activity of protein quality control regulating transcription factors.

#### *Insulin/IGF1 pathway*

The insulin/insulin growth factor 1 (IGF1) signaling (IIS) pathway is one of the most important pathway for regulation protein homeostasis under non-stress conditions (171). Reduced IIS can prolong life span and increase stress resistance, including resistance to the chronic expression of aggregation-prone proteins (172-174).

There are two major protein quality control-related transcription factors, HSF1 and FOXO1 that are regulated by the IIS pathway (171, 175). The basal activities of both of these are inhibited when the IIS is active, while basal activities of both HSF1 and FOXO1 are induced when IIS is inactivated (e.g. under caloric restriction or upon receptor deletion) (176-179). Intriguingly, the sole activation of either HSF-1 or FOXO1 (with or without stress) suffices to cause longevity and resistance to chronic stress (173, 180). Whereas, the effects of HSF-1 activation are considered to be due to activation of several heat shock proteins, but precise understanding how FOXO1 mediates its effects is yet not well understood.

FOXO1 is a member of family of Forkhead box protein. Its ortholog in *C. elegans*, DAF16, has been suggested to up-regulate the small HSPs, HSP-16 (181, 182). Some member of the small HSPs

family, for example, HspB7 can prevent polyQ aggregation and rebalance the protein homeostasis (54, 183). Moreover, the mammalian FOXO1 itself has been linked to autophagy which is the major pathway for aggregation clearance (184). In *C. elegans*, DAF-16 (the homolog of FOXO1) is also related to extended life span and the protection against polyQ aggregation in a sHSP related manner (181). However, whether and how FOXO1 plays a role in resistance to chronic stress like the handling of misfolded proteins in mammalian cells has remained unexplored.

HSF1 (heat shock factor 1) is another transcriptional regulator downstream of IGF1 and is a center player of the HSR (185). HSF1 responds to multiple stress factors and upregulates the expression of a series of HSP family members such as HSP70, HSP90 and some HSPBs, therefore, contributes to the protein homeostasis by mediating chaperone mediated refolding and protein degradation (185). However, although clearly having protective effects in acute stress (186), the role of HSF-1 in chronic stress is less clear. Whereas HSF-1 activation is protective in both of *C. elegans* (180) and *Drosophila* (187), it seems less or ineffective in mammalian cell (187) and animal systems (188). HSF-1 regulated HSP like HSPA1A that are rate limiting in protecting in acute stress (189-192) and do not reduce polyQ aggregation (105, 193, 194) or only seem to ameliorate the toxic consequences of aggregation (194).

Within the family of small HSP, those regulated by HSF-1 (HSPB1, HSPB5, HSPB8) have no or only minor effects on polyQ aggregation (54, 195). However, a non-canonical HspB members, HspB7, that is not under regulation of HSF-1 has been found to reduce polyQ aggregation (183). Unlike most HspBs, HspB7 does not form oligomers and seemed to work independently of Hsp70 machinery and does not seem to act in acute stresses conditions, as it e.g. does not assist in refolding of heat-unfolded luciferase (54). In chapter 3, studies how HspB7 acts in chronic stress conditions polyQ aggregation.

#### *Insulin/IGF2 pathway*

Besides IGF1, mammalian cells express a second insulin like growth factor, IGF2 which is less well studied. IGF2 has been mainly connected with the development of cancer and cardiovascular diseases (196). IGF2 preferentially binds to IGF type 2 receptor (IGF2R) (197), but also binds to the IGF type 1 receptor (IGF1R) (197). Recently, the group of Claudio Hetz demonstrated that the ER-related transcription factor XBP1 is IGF2 regulated. Inversely, IGF2 is one of the major genes upregulated in XBP1 knockout mice/cells. Most strikingly, IGF2 was also protective in a model of Alzheimer's disease (198). How IGF2 exerts these effects and whether or not these are similar to or overlapping with effects related to IGF1 has yet to be established and will be addressed in chapter 5.



**The outline of this study**

The aim of this thesis is to study the regulation of protein homeostasis in acute and chronic stress. For acute stress, we tried to evaluate the possibility of using aspirin pre-treatment to reduce heat stress injury on chicken myocardial cells in breeding industrial. The hypothesis is that appropriate pre-treatments with ASA can activate the expression of HSPs in the myocardial cells of chicken in a non-toxic manner such that the myocardial cells are protected against temperature induced cytoskeletal collapse.

For chronic stress, we addressed how cells can handle the chronic expression of a mutant Huntingtin (mHtt) protein and prevent its toxic aggregation. We focused on HSPB7, a non-canonical, non-HSF-1 regulated small HSP which previously was found to be the best of all human HSPB members in suppressing polyQ aggregation (54). First, we unraveled the molecular mechanism behind this specific functionality of the HSPB member. Second, we asked whether any small HSPs could be one of the target proteins of the Insulin/IGF pathway that is responsible for the protective effects of these pathways in polyQ diseases or whether other mechanisms may (also) underlie their actions.

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